THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 52, Issue of December 27, pp. 50348–50354, 2002
Printed in U.S.A.
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

The Endocannabinoid System Protects Rat Glioma Cells Against HIV-1 Tat Protein-induced Cytotoxicity

MECHANISM AND REGULATION*

Received for publication, July 17, 2002, and in revised form, October 14, 2002
Published, JBC Papers in Press, October 17, 2002, DOI 10.1074/jbc.M207170200

Giuseppe Esposito‡, Alessia Ligresti§, Angelo A. Izzo‡, Tiziana Bisogno§, Menotti Ruvo¶, Massimo Di Rosa‡, Vincenzo Di Marzo§‖, and Teresa Iuvone‡**

From the ‡Department of Experimental Pharmacology, University of Naples "Federico II", Via D. Montesano 49, 80131 Naples, Italy, the §Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, 80078 Pozzuoli, Naples, Italy, and the ¶Istituto di Biostrutture e Bioimmagini, Consiglio Nazionale delle Ricerche, Via Mezzocannone 6, 80134 Naples, Italy

Cannabinoids modulate nitric oxide (NO) levels in cells of the central nervous system. Here we studied the effect of cannabinoid CB_1 and CB_2 receptor agonists on the release of NO and cell toxicity induced by the human immuno-deficiency virus-1 Tat protein (HIV-1 Tat) in rat glioma C6 cells. The CB_1 and CB_2 agonist WIN 55,212–2 inhibited the expression of inducible NO synthase (iNOS) and NO release caused by treatment of C6 cells with HIV-1 Tat and interferon-γ (IFN-γ). The effect of WIN 55,212–2 was uniquely due to CB_1 receptors, as shown by experiments carried out with selective CB_1 and CB_2 receptor agonists and antagonists. CB_1 receptor stimulation also inhibited HIV-1 Tat + IFN-γ-induced and NO-mediated cell toxicity. Moreover, cell treatment with HIV-1 Tat + IFN-γ induced a significant inhibition of CB_1, but not CB_2, receptor expression. This effect was mimicked by the NO donor GSNO, suggesting that the inhibition of CB_1 expression was due to HIV-1 Tat + IFN-γ-induced NO overexpression. HIV-1 Tat + IFN-γ treatment also induced a significant inhibition of the uptake of the endocannabinoid anandamide by C6 cells with no effect on anandamide hydrolysis. These findings show that the endocannabinoid system, through the modulation of the L-arginine/NO pathway, reduces HIV-1 Tat-induced cytotoxicity, and is itself regulated by HIV-1 Tat.

Tat (trans-activating proteins) are early RNA-binding proteins that regulate gene transcription. Human immunodeficiency virus-1 Tat (HIV-1 Tat)[1] is a viral protein of 86 amino acids encoded by two exons, which plays a crucial role in the replication of the HIV-1 virus (1, 2). The sequence containing the first 72 amino acids, encoded by exon 1, exhibits the full trans-activating activity, whereas the exon 2-encoded 14 amino acid C-terminal sequence is important for the binding to membrane integrins (3). During the initial phase of infection, large amounts of HIV-1 Tat, together with other regulatory proteins, are synthesized and released from HIV-1-infected cells (4, 5). HIV-1 Tat can then translocate across the cell membrane and localize in the nucleus of uninfected cells where it drives virus replication (3). In addition, HIV-1 Tat modulates the expression of many genes regulating important functions such as cell survival and growth, inflammation, and angiogenesis (2). Frequently HIV-1 infection is associated with brain damage (6) characterized by cerebral atrophy, neuronal loss, gliosis, infiltration of inflammatory cells, and microglial activation (7, 8). All these pathological changes are accompanied by severe cognitive and motor abnormalities; neurological symptoms begin with difficulty in concentration, forgetfulness, and behavioral abnormalities, collectively known as AIDS dementia complex, subsequently progressing to florid dementia, motor impairment, coma, and death (9, 10).

Marijuana, a mixture of the leaves and flowering tops of *Cannabis sativa,* has been used for centuries by mankind to alleviate the symptoms of a wide range of health problems, including mental disorders (11). The pharmacological effects of psychoactive cannabinoids, including Δ^9-tetra-hydrocannabinol, the main psychotropic component of marijuana, depend on the interaction with specific G protein-coupled receptors. Two cannabinoid receptors, named CB_1 and CB_2, have been so far identified (for review, see Ref. 12). CB_1 receptors are mainly expressed in the central and peripheral nervous systems (13, 14), whereas CB_2 receptors have been identified in cells of the immune system (15) and in C6 cells, a rat glioma cell line (16). Anandamide (arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG) have been proposed as the endogenous ligands of cannabinoid receptors (17–20). The endogenous cannabinoid (endocannabinoid) system, *i.e.* the cannabinoid receptors and their endogenous ligands, seems to play a role in neuroprotection, as suggested by the up-regulation of CB_1 receptor expression and the elevated concentrations of endocan-

* This work was supported by Grant 3933 from Ministero Università e Ricerca Scientifica e Tecnologica (to V. D. M). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence may be addressed: Via Campi Flegrei 34, Comprensorio Olivetti, Ed. 70, 80078 Pozzuoli, Naples, Italy. Tel.: 39-081-8675093; Fax: 39-081-8041770; E-mail: vdimarzo@icmib.na.cnr.it.

** To whom correspondence may be addressed: Via D. Montesano 49, 80131 Naples, Italy. Tel.: 39-081-678429; Fax: 39-081-678403; E-mail: iuvone@unina.it.

[1] The abbreviations used are: HIV-1 Tat, human immunodeficiency virus-1 Tat; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; anandamide, arachidonoylethanolamide, AEA; ACEA, arachidonyl-2′-chloroethylamide/(all *Z*)-N-(2-chloroethyl)-5,8,11,14-eicosatetraenamide; JWH-015, (2-Methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; WIN 55,212–2, (R)-(+)-[2,3 dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone; GSNO, S-nitroso-glutathione; SR141716A, [(N-piperidin-1-yl)]-5-(4-chlorophenyl)-1–2-4-dichlorophenyl)-4 methyl-1*H*-pyrazole-3-carboxamide hydrochloride; SR144528, (*N*-[-1S-endo-1,3,3-trimethyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide; Fmoc, N-(9-fluorenyl)-methoxycarbonyl; HPLC, high pressure liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; nt, nucleotide; ANOVA, analysis of variance.

This paper is available on line at http://www.jbc.org

This is an Open Access article under the CC BY license.

nabinoids in some *in vivo* models of neurodegeneration and brain injury (21–24). Recent findings have demonstrated that cannabinoids are able to down-regulate the production of nitric oxide (NO), a short-lived mediator produced by the L-arginine/NO pathway, in macrophages (25, 26) and in rat microglial and glial cells (27, 28). In the brain, NO is produced by both the constitutive isoform of NO synthase (cNOS) found primarily in neurons (29) and astroglial cells (30), and the inducible isoform of NO synthase (iNOS) expressed in stimulated astrocytes (31), in a human astrocytoma cell-line (32), and in rat C6 cells (28). NO produced by cNOS in picomolar amounts mediates several physiological effects in the central nervous system, such as neurotransmission (33, 34), whereas NO generated by iNOS in nanomolar amounts under pathological conditions is a cytotoxic mediator involved in several central nervous system disorders, including inflammatory, infectious, traumatic, and degenerative diseases (35, 36).

Recently, activated glial cells have also been proposed to play an active role in many neurodegenerative pathologies (37–39) through the production and release of several pro-inflammatory and cytotoxic mediators (40, 41), including nitric oxide (28).

The aim of this study was to evaluate the role of the endocannabinoid system in the control of NO release and cytotoxicity both induced by HIV-1 Tat protein in C6 cells and to assess whether this insult affects endocannabinoid action and homeostasis. Our study casts further light on the role of NO as a potential neurotoxic mediator and on the molecular basis of cannabinoid neuroprotective actions.

EXPERIMENTAL PROCEDURES

Tat Protein Chemical Synthesis—HIV-1 Tat (1–86) was chemically synthesized by step-by-step solid phase peptide synthesis following the Fmoc/1-hydroxybenzotriazole/*N*,*N*′-dicyclohexylcarbodiimide methodology as previously described (42). The crude material isolated after trifluoroacetic acid cleavage was purified by semi-preparative reversed-phase-HPLC and characterized by analytical RP-HPLC (purity > 95%), time-of-flight matrix-assisted laser desorption ionization, and electrospray ionization mass spectrometry. The protein was used throughout the experiments without further manipulations dissolved in pyrogen-free distilled water.

Cell Culture and Treatments—C6 rat glioma cells (American Type Culture Collection CCL-107) were grown in Petri dishes (10 cm diameter) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin at 37 °C in 5% CO_2/95% air. Confluent cells were detached by trypsin/EDTA (1:25 v/v), counted, and plated in 24 multi-well plates at a density of 2.5×10^5 cells/well and allowed to adhere for 24 h at 37 °C. Thereafter, the medium was replaced with fresh medium to a final volume of 1 ml, and cells were treated with interferon-γ (IFN-γ, 100 units/ml) for 16–18 h. After this time cells were stimulated with HIV-1 Tat protein (500 ng/ml) for a further 24 h according to Polazzi *et al.* (43). In preliminary experiments, the concentration-dependent effect of HIV-1 Tat (100–500-1000 ng/ml) was investigated, and 1000 ng/ml of this protein, in addition to IFN-γ (100 units/ml), was chosen as the stimulus to be used in the following experiments. Test compounds were the non-selective cannabinoid agonist WIN 55,212–2 (10^{-9}–10^{-6} M) or the selective CB_1 receptor agonist ACEA or CB_2 selective agonist JWH-015 (10^{-9}–10^{-6} M) were added concomitantly to Tat protein. The selective CB_1 antagonist SR141761A (10^{-9}–10^{-6} M) and the selective CB_2 antagonist SR144528 (10^{-9}–10^{-6} M) were given 30 min before the agonists. All the test compounds were given in 100 μl of volume. In some experiments, to test the hypothesis that NO may regulate cannabinoid receptor expression, cells were incubated for 24 h in the presence of the NO donor GSNO (10^{-4} M).

Nitrite Assay—NO production was measured as the stable metabolite nitrite (NO_2^-) accumulated in the incubation medium of C6 cells after 24 h following Tat + IFN-γ addition, using a spectrophotometric assay based on Griess reaction as previously described (44).

Preparation of Cytosolic Fractions—Extracts of C6 cells stimulated for 24 h with Tat (100–500-1000 ng/ml) + IFN-γ (100 units/ml) in the presence or absence of WIN 55,212,2 (10^{-9}–10^{-6} M) and with WIN 55,212–2 (10^{-7} M) plus both CB_1 and CB_2 receptor antagonists (10^{-7} M), or in other experiments extracts of C6 treated for 24 h with the NO donor, GSNO (100 μM), were prepared as previously described (28). Briefly, harvested cells (2×10^7) were washed twice with ice-cold phosphate-buffered saline and centrifuged at $180 \times g$ for 10 min at 4 °C. The cell pellet was resuspended in 100 μl of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulphonyl fluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated in ice per 15 min. The cells were lysed by rapid passage through a syringe needle five or six times, and the cytoplasmic fraction was then obtained by centrifugation at $13000 \times g$ for 1 min.

Western Blot Analysis—Immunoblotting analysis of iNOS protein and CB_1 and CB_2 receptors were performed on cytosolic fraction of C6 cells treated as above described. Cytosolic fraction proteins were mixed with gel loading buffer (50 mM Tris/10% SDS/10% glycerol 2-mercaptoethanol/2 mg bromphenol per ml) in a ratio of 1:1, boiled for 3 min, and centrifuged at $10,000 \times g$ for 10 min. Protein concentration was determined, and equivalent amounts (50 μg) of each sample were electrophoresed in a 10% discontinous polyacrilamide minigel. The proteins were transferred onto nitrocellulose membrane, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4 °C overnight with 10% nonfat dry milk in phosphate-buffered saline and then incubated with mouse anti-iNOS antibody (1:1000) (Transduction Laboratories) and rabbit polyclonal anti-CB_1 or anti-CB_2 antibody (1:500) (Cayman) for 1 h at room temperature. The membranes were washed three times with 1% Triton X-100 in phosphate-buffered saline and then incubated with anti-mouse or anti-rabbit immunoglobulins coupled to peroxidase (1:2000) (Amersham Biosciences). The immune complexes were developed using enhanced chemiluminescence detection reagents (Amersham Biosciences) according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of iNOS, CB_1, and CB_2 on x-ray film were scanned and densitometrically analyzed with a GS-700 imaging densitometer.

MTT Viability Assay—Cell viability was measured by MTT assay, according to Esposito *et al.* (45). The cells were plated in 96-well culture plates at the density of 5×10^3 cells/ml/well and allowed to adhere at 37 °C. Thereafter, the medium was replaced with 50 μl of fresh medium, and cells were incubated with Tat (1000 ng/ml) + IFNγ (100 units/ml) for 24 h in the presence or absence of WIN 55,212,2 (10^{-9}–10^{-6} M) and with WIN 55,212–2 (10^{-7} M) plus either CB_1 or CB_2 receptor antagonists (10^{-9}–10^{-6} M). After this time, MTT (25 μl) was added in each well, and cells were incubated for additional 3 h at 37 °C. Afterward, cells were lysed and the dark blue crystals solubilized with 125 μl of a solution containing 20% sodium dodecylsulphate in 1:1 deionized water/dimethylformamide, with an adjusted pH of 4.5, and wells were incubated overnight. The cell viability in response to the treatment with test compounds was measured by a spectrophotometric assay at 620 nm by detecting the complex MTT-formazan salt-conjugated and was expressed as % of cell viability = (OD treated/OD control) × 100.

Total RNA Isolation and RT-PCR Analysis—Total RNA from C6 cells was extracted using Trizol reagent according to the manufacturer's recommendations (Invitrogen). Following extraction, RNA was precipitated using ice-cold isopropanol, resuspended in diethyl pyrocarbonate (Sigma)-treated water, and its integrity was verified following separation by electrophoresis into a 1% agarose gel containing ethidium bromide. RNA was further treated with RNase-free DNase I (Ambion DNA-free™ kit) according the manufacturer's recommendations to digest contaminating genomic DNA and to subsequently remove the DNase and divalent cations.

The expression of mRNAs for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), CB_1, and CB_2 receptors was examined by reverse transcription coupled to the polymerase chain reaction (RT-PCR). Total RNA was reverse-transcribed using oligo dT primers. DNA amplifications were carried out in PCR buffer (Q-Biogen) containing 2 μl of cDNA, 500 μM deoxynucleotide tris-phosphate, 2 mM $MgCl_2$, 0.8 μM of each primer, and 0.5 units *Taq* polymerase (Q-Biogen). The thermal reaction profile consisted of a denaturation step at 94 °C for 1 min, annealing at 60 °C for 1 min, and an extension step at 72 °C for 1 min. A final extension step of 10 min was carried out at 72 °C. The PCR cycles ranging from 20 to 40 were observed to be optimal and in the linear portion of the amplification curve (data not shown). Reaction was performed in a PE Gene Amp PCR System 9600 (PerkinEmer Life Sciences). After reaction, the PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide for UV visualization.

The specific human oligonucleotides were synthesized on the basis of cloned rat cDNA sequences of GAPDH, CB_1, and CB_2. For GAPDH, the primers sequences were 5′-CCCTTCATTGACCTCAACTACATGGT-3′

(nt 208–233; sense) and 5′-GAGGGCCATCCACAGTCTTCTG-3′ (nt 655–677; antisense). The CB_1 sense and antisense primers were 5′-GATGTCTTTGGGAAGATGAACAAGC-3′ (nt 365–373) and 5′-AGACGTGTCTGTGGACACAGACATGG-3′ (nt 460–468), respectively. For CB_2, the primer sequences were 5′-CCCATGCAGGA(G/T)TACATGATCCTGAG-3′ (nt 20–29; sense) and 5′-CTCCGC(A/C)G(A/G)AAGCCCTC(A/G)TAC-3′ (nt 64–70; antisense). The expected sizes of the amplicons were 470 bp for GAPDH, 309 bp for CB_1, and 150 bp for CB_2. The GAPDH housekeeping gene expression was used to evaluate any variation in the RNA content and cDNA synthesis in the different preparations. No PCR products were detected when the reverse transcriptase step was omitted (data not shown).

Anandamide Uptake by C6 Cells—Confluent C6 cells in 6-well dishes after treatment with either vehicle or HIV-1 Tat (1000 ng/ml) + IFN-γ (100 units/ml) were incubated for either 10 or 20 min with [^{14}C]anandamide (5 mCi/mmol, 20,000 cpm, 4 μM) in serum-free culture medium at either 37 or 4 °C. After the incubation the amount of residual [^{14}C]anandamide in the culture medium was extracted with chloroform/methanol 2:1 (by volume), quantified by liquid scintillation counting, and taken as a measure of the [^{14}C]anandamide taken up by cells (see 46). Nonspecific uptake was measured from the 4 °C incubations and subtracted from total uptake.

Anandamide Hydrolysis by C6 Cell Membranes—Mitochondrial membranes were prepared from C6 cells treated with either vehicle or HIV-1 Tat (500 ng/ml) + IFN-γ (100 units/ml), as described previously (47), and used as a source of fatty acid amide hydrolase (FAAH, also known as anandamide amidohydrolase) to carry out enzymatic hydrolysis assays with [^{14}C]anandamide (5 mCi/mmol, 40000 cpm, 10 μM) (47). [^{14}C]Ethanolamine produced from [^{14}C]anandamide hydrolysis was extracted from the incubation mixtures by extraction with chloroform/methanol 2:1 (by volume), quantified by liquid scintillation counting, and used to measure the rate of the enzymatic reaction.

Materials—All chemicals for solid phase peptide synthesis (Fmoc-protected aminoacids, resins, and condensing agents) were from Novabiochem (Laufelfingen, Switzerland) and Applied Biosystem (Foster City, CA); solvents for HPLC analysis and purification and trifluoroacetic acid and scavengers for peptide cleavage were from Sigma. Columns for protein characterization and purification were from Phenomenex (Torrance, CA).

All the materials for cell culture were purchased from BioWhittaker (Caravaggio, BG, Italy). Fetal calf serum was from Hyclone. IFN-γ was from PBL Biomedical Laboratories (Vinci-Biochem, Vinci, Italy). WIN 55,212–2, ACEA, and JWH 015 were from Tocris Cookson (Bristol, UK). SR141716A and SR144528 were a kind gift from Drs. Madeleine Mossè and Francis Barth from SANOFI-Recerche.

Statistical Analysis—Results were expressed as the mean ± S.E. of n experiments. Statistical analysis was determined with ANOVA and multiple comparisons were performed by Bonferroni's test, with $p < 0.05$ considered significant.

RESULTS

Concentration-dependent Effect of HIV-1 Tat Protein on Nitrite Production and iNOS Expression—Unstimulated C6 cells produced only low amounts of nitrite (2.5 ± 0.2 nmol/10^6 cells). The effect of HIV-1 Tat on nitrite production was studied by incubation of C6 cells with IFN-γ (100 units/ml) for 16–18 h, after which the cells were stimulated further for 24 h with HIV-1 Tat. The incubation of cells with IFN-γ (100 units/ml) alone for 16–18 h did not induce a significant increase of nitrite production (7.6 ± 1.5 nmol/10^6 cells) in comparison to unstimulated cells; stimulation with Tat protein (100–500-1000 units/ml) for a further 24 h resulted in a significant and concentration-dependent increase of nitrite production in the cell medium (9.3 ± 0.8, 19.2 ± 1.3, and 28.0 ± 2.5 nmol/10^6 cells, respectively). The presence of iNOS in the cytosolic fractions was investigated by immunoblotting analysis. A basal level of iNOS was detectable in the cytosolic fractions of unstimulated cells; after 16–18 h of IFN-γ (100 units/ml) treatment iNOS expression slightly increased, whereas HIV-1 Tat protein (100–500-1000 ng/ml) stimulation of cells for a further 24 h markedly increased iNOS expression in a concentration-dependent fashion (Fig. 1).

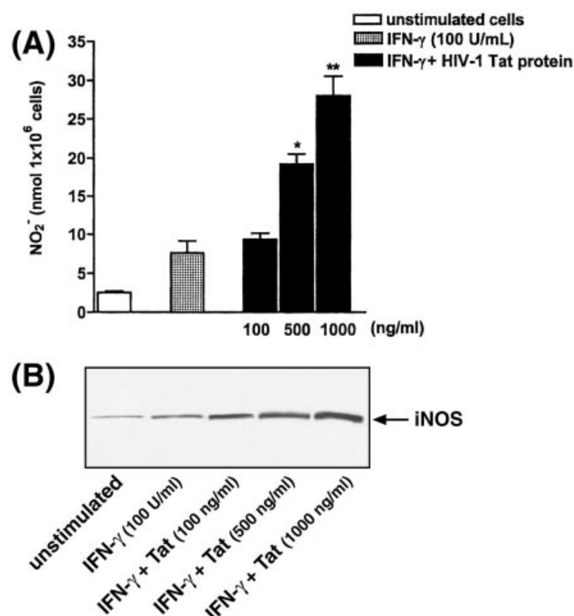

FIG. 1. **Effect of HIV-1 Tat protein on nitrite (NO_2^-) production (*A*) and iNOS protein expression (*B*) in C6 rat glioma cells.** Cells were cultured for 16–18 h in the presence of IFN-γ (100 units/ml) and thereafter, HIV-1 Tat protein (100–500-1000 ng/ml) was added and cells incubated for further 24 h. Equal amounts of protein (50 μg/lane) of proteins were analyzed by Western blot, using a specific anti-iNOS monoclonal antibody. The *bars* represent the mean ± S.E. of five experiments in triplicate. Western blot of iNOS is representative of three separate experiments. No significant increase of NO_2^- production by HIV-1 Tat alone was observed (not shown). **, $p < 0.01$; *, $p < 0.05$ *versus* unstimulated cells.

Effect of WIN 55,212–2 and the Selective CB_1 and CB_2 Receptor Antagonists on Nitrite Production and iNOS Expression—The concomitant addition of the cannabinoid mimetic WIN 55,212–2 (10^{-9}–10^{-6} M) to cells stimulated with HIV-1 Tat (1000 ng/ml) + IFN-γ (100 units/ml), resulted in a significant and concentration-dependent inhibition of nitrite production respectively from 25.2 ± 1.2 to 21.7 ± 2.0, 17.3 ± 1.5, and 13.4 ± 1.0 nmol/10^6 cells, in confront to stimulated cells (28.0 ± 2.5 nmol/10^6 cells) (Fig. 2).

The inhibitory effect of WIN 55,212–2 (10^{-7} M) on nitrite production was reversed in a concentration-dependent fashion by the selective cannabinoid CB_1 receptor antagonist SR141716A (10^{-9}–10^{-6} M) added to the cells 15 min before WIN 55,212–2 (19.5 ± 1.6, 21.0 ± 1.2, 24.0 ± 1.9, and 26.5.0 ± 2.3 nmol nitrite, respectively) in comparison to WIN 55,212–2 10^{-7} M (17.3 ± 1.5 nmol nitrite). Conversely, the selective cannabinoid CB_2 receptor antagonist SR144528 (10^{-9}–10^{-6} M) was ineffective (17.0 ± 1.8, 17.4 ± 1.4, 16.9 ± 2.1, and 16.0 ± 1.3 nmol nitrite) (Fig. 3, *A* and *B*). SR141716A and SR144528, given alone, did not affect nitrite production by C6 cells (data not shown).

Treatment of cells with WIN 55,212–2 (10^{-9}–10^{-6} M) resulted in a strong and dose-dependent decrease of iNOS protein expression in the cytosolic fractions. The inhibitory effect of WIN 55,212–2 (10^{-7} M) on iNOS protein expression was reversed by SR141716A (10^{-7} M) (75%) but not by SR144528 (10^{-7} M) (Fig. 4).

Effect of Selective CB_1 and CB_2 Agonist on Nitrite Production—The addition of the selective CB_1 receptor agonist, ACEA (10^{-9}–10^{-6} M) to C6 cells stimulated by HIV-1 Tat (1000 ng/ml) + IFN-γ (100 units/ml) induced a significant and concentration-dependent inhibition of nitrite production up to 71.4% (22.6 ± 2.0, 19.5 ± 1.7, 13.0 ± 1.0, and 8.0 ± 0.5 nmol/10^6 cells, respectively) in comparison to stimulated cells (28.0 ± 2.5 nmol/10^6 cells). In contrast, the addition of the CB_2 selective agonist, JWH-015 (10^{-6}–10^{-9} M), to stimulated cells did not have any effect on nitrite production (27.0 ± 1.0, 26.0 ± 2.0,

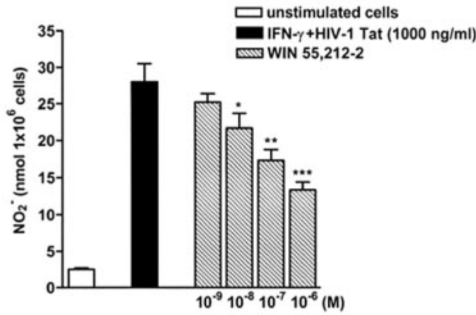

FIG. 2. **Effect of WIN 55,212–2 on IFN-γ (100 units/ml) + HIV-1 Tat protein (1000 ng/ml)-induced nitrite (NO_2^-) production in C6 rat glioma cells.** *Open bars* represent control cells (unstimulated cells), *filled bars* represent IFN-γ + Tat-stimulated cells, and *hatched bars* represent cells treated with WIN 55,212–2 (10^{-9}–10^{-6} M) added concomitantly to IFN-γ + Tat. Each *bar* represents the mean ± S.E. of five experiments in triplicate. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$ *versus* stimulated cells.

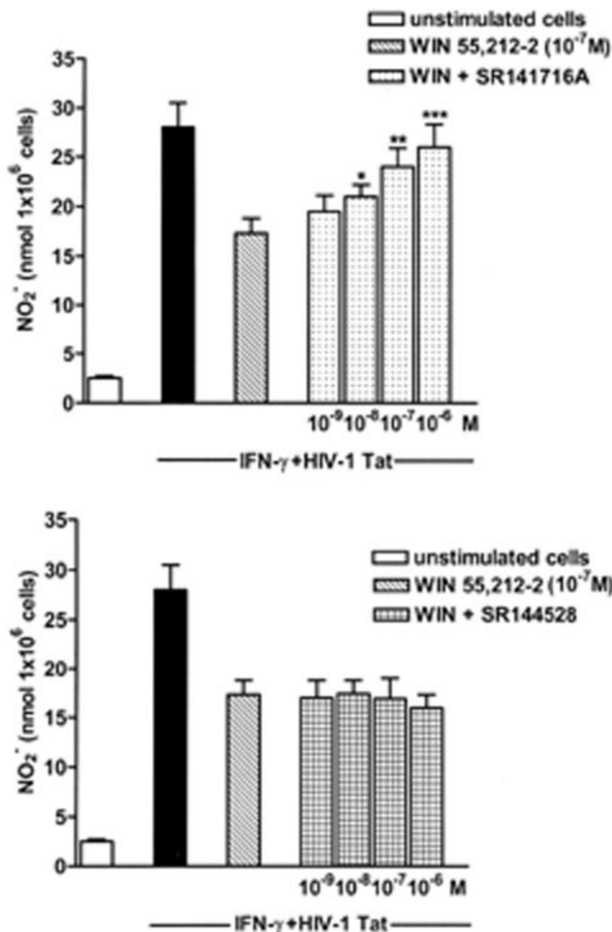

FIG. 3. **Effect of cannabinoid receptor antagonists SR141716A (CB_1 antagonist) (*A*) and SR144528 (CB_2 antagonist) (*B*) on WIN 55,212–2-mediated inhibition of IFN-γ (100 units/ml) + HIV-1 Tat protein (1000 ng/ml)-induced nitrite production in C6 rat glioma cells.** *Open bars* represent control cells (unstimulated cells), *filled bars* represent IFN-γ + Tat-stimulated cells, and *hatched bars* represent cells treated with WIN 55,212–2 (10^{-7} M) added concomitantly to IFN-γ + Tat. Both CB_1 and CB_2 antagonists (10^{-9}–10^{-6} M) were added 15 min before the stimulus to the cells treated with WIN 55,212–2 (10^{-7} M). Each *bar* represents the mean ± S.E. of four experiments in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* WIN 55,212–2 treated cells.

29.0 ± 1.9, and 27.0 ± 2.0 nmol/10^6 cells) by stimulated cells (Fig. 5, *A* and *B*).

HIV-1 Tat-induced Cytotoxicity—Incubation of C6 cells with

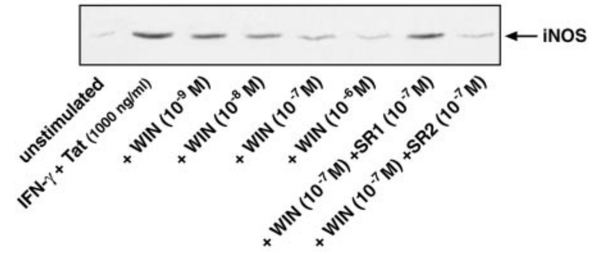

FIG. 4. **Effect of the cannabinoid receptor agonist WIN 55,212–2 and both CB_1 receptor antagonist SR141716A and CB_2 receptor antagonist SR144528 on iNOS protein expression in C6 cells stimulated with INF-γ (100 units/ml) + HIV-1 Tat protein (1000 ng/ml).** Cells were stimulated for 24 h with IFN-γ (100 units/ml) + HIV-1 Tat protein (1000 ng/ml) in presence of WIN 55,212–2 (10^{-9}–10^{-7} M) or WIN 55,212–2 (10^{-7} M) plus CB_1 or CB_2 antagonists (10^{-7} M). Equal amounts of protein (50 μg/lane) were analyzed by Western blot, using a specific anti-iNOS monoclonal antibody. Western blot of iNOS is representative of three separate experiments.

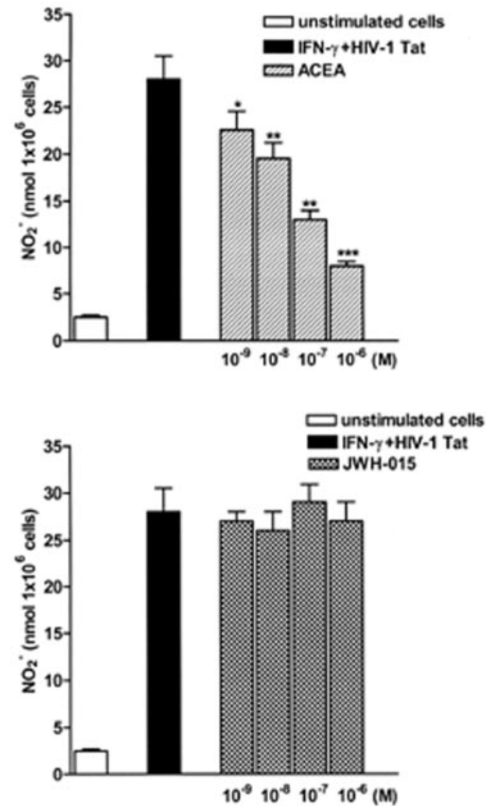

FIG. 5. **Effect of the CB_1 selective agonist ACEA and the CB_2 selective agonist JWH-015 on nitrite (NO_2^-) production at 24 h in C6 rat glioma cells, stimulated with IFN-γ (100 units/ml) + HIV-1 Tat protein (1000 ng/ml).** *Open bars* represent control cells (unstimulated cells), *filled bars* represent IFN-γ + Tat-stimulated cells, *hatched bars* represent ACEA (10^{-9}–10^{-6} M) treated cells, and *cross bars* represent JWH-015 (10^{-9}–10^{-6} M) treated cells. Each *bar* represents the mean ± S.E. of four experiments in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* stimulated cells.

IFN-γ for 16–18 h caused a slight, not statistically significant decrease of cell viability, *i.e.* 91.0 ± 1.1% viability in comparison to untreated cells (100% viability). Treatment with HIV-1 Tat protein (1000 ng/ml) for further 24 h decreased cell viability up to 60.0 ± 1.9% of control viability. WIN 55,212–2 (10^{-10}–10^{-6} M) protected cells from death also at low concentrations (65.2 ± 4.2, 68.0 ± 2.5, 71.0 ± 2.8, 80.5 ± 2.0, and 81.5 ± 1.6% of control viability, respectively). The protective effect of WIN 55–212,2 (10^{-7} M) was concentration-dependently reversed by SR141716A, but not by SR144528 (Fig. 6).

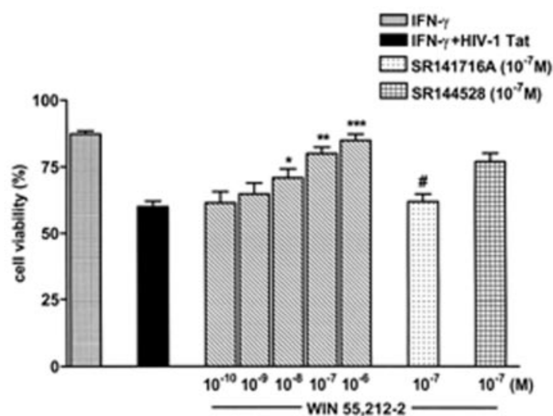

FIG. 6. **Effect of cannabinoid receptor agonist, WIN 55,212–2, and both CB_1 receptor antagonist, SR141716A, and CB_2 receptor antagonist, SR144528, on cell viability evaluated in C6 cells stimulated with INF-γ + HIV-1 Tat protein.** Cells were incubated for 24 h with IFN-γ (100 units/ml) + HIV-1 Tat protein (1000 ng/ml) in the presence of WIN 55,212–2 (10^{-10}–10^{-6} M) or WIN 55,212–2 (10^{-7} M) plus CB_1 or CB_2 antagonists (10^{-7} M). The cell viability in response to the treatment with test compounds was measured by a spectrophotometric assay at 620 nm by detecting the complex MTT-formazan salt-conjugated, and was expressed as % of cell viability = (OD treated/OD control) × 100. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* stimulated cells. #$p < 0.05$ *versus* WIN 55,212–2 (10^{-7} M)-treated cells.

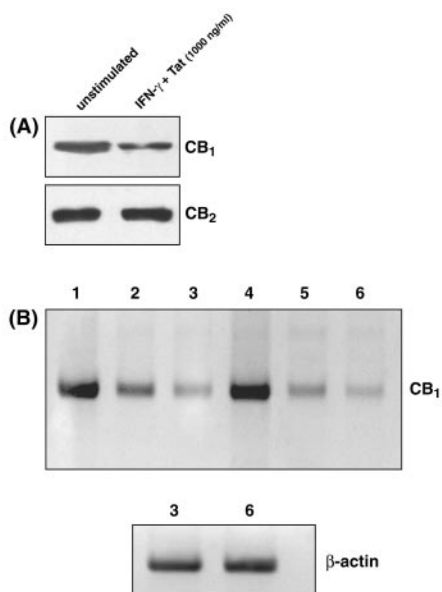

FIG. 7. **Effect of IFN-γ + HIV-1 Tat protein on CB_1 and CB_2 receptor protein expression.** *A*, Western blot analysis of CB_1 and CB_2 receptor were performed on cytosolic fraction of both untreated C6 cells or cells treated with IFN-γ (100 units/ml) + HIV-1 Tat protein (1000 ng/ml) for 24 h. Equal amounts of protein (50 μg/lane) were analyzed using specific rabbit polyclonal anti-CB_1 or anti-CB_2 antibody. *B*, quantitative RT-PCR analysis of RNA from C6 cells treated with either vehicle (*lanes 1–3*) or IFN-γ (100 units/ml) + HIV-1 Tat (1000 ng/ml) (*lanes 4–6*). *Bands* represent the amplicons obtained from 40 (*lanes 1* and *4*), 30 (*lanes 2* and *5*), or 20 (*lanes 3* and *6*) cycles of PCR carried out with oligoprobes for CB_1 receptor (*upper panel*) or β-actin (*lower panel*) on the cDNA obtained from reverse transcription of RNA from vehicle (*lanes 1–3*) or HIV-1 Tat + IFN-γ (*lanes 4–6*) treated C6 cells. Bands were of the expected size for CB_1 (309 bp) and GAPDH (470 bp) amplicons.

Down-regulation of CB_1, but Not CB_2, Receptors by HIV-1 Tat—Treatment of C6 cells with HIV-1 Tat + IFN-γ led to a significant reduction of CB_1, but not CB_2, receptor protein, as assessed by Western immunoblotting (Fig. 7*A*). Quantitative RT-PCR analysis showed that the inhibitory effect was exerted

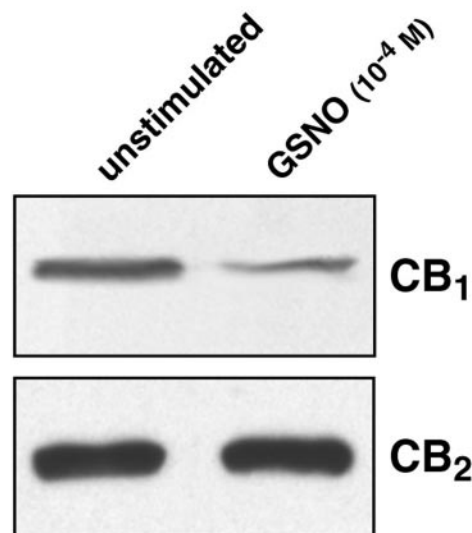

FIG. 8. **Effect of NO donor, GSNO, on CB_1 and CB_2 receptor protein expression.** Western blot analyses of CB_1 and CB_2 receptor were performed on cytosolic fraction of both untreated C6 cells or cells treated with GSNO (10^{-4} M) for 24 h. Equal amounts of protein (50 μg/lane) were analyzed using specific rabbit polyclonal anti-CB_1 or anti-CB_2 antibody. Western blot is representative of three separate experiments.

at the transcriptional level, because the amounts of CB_1, but not CB_2, mRNA transcripts were significantly reduced in cells treated with IFN-γ + HIV-1 Tat (Fig. 7*B* and data not shown).

Effect of the NO-donor, GSNO, on CB_1 and CB_2 Receptor Protein Expression—Treatment of C6 cells with GSNO (10^{-4} M), an NO donor, for 24 h resulted in a significant decrease of CB_1, but not CB_2, receptor expression (47.3% of the expression respective to untreated control cells, as shown by densitometric analysis) (Fig. 8).

Effect of HIV-1 Tat on Anandamide Inactivation by C6 Cells—Treatment of C6 cells with IFN-γ + HIV-1 Tat led to a significant 16.5% reduction in the uptake of $[^{14}C]$anandamide as compared with untreated cells after 10 min incubations (from 462.6 ± 19.0 to 386.2 ± 23.8 pmol/10^6 cells, $p < 0.05$ by ANOVA). By contrast, the same treatment did not influence significantly the capability of membranes prepared from C6 cells to hydrolyze $[^{14}C]$anandamide (from 138.7 ± 12.0 to 117.3 ± 3.0 pmol/mg protein, $p > 0.05$ by ANOVA).

DISCUSSION

In this study we show that the HIV-1 Tat protein is able to induce iNOS protein expression and nitrite production in the astroglial-derived cell line, C6 (rat glioma cells), pre-incubated with IFN-γ. Our findings are in agreement with the results reported by Polazzi *et al.* (43) in rat microglial cells. Overproduction of nitrite by the activated glia may amplify an inflammatory process leading to neuronal death and, in turn, to neurodegeneration, such as in HIV-related dementia complex, frequently observed in HIV-affected patients (48).

The results of our study demonstrate that HIV-1 Tat-induced iNOS protein expression and nitrite production in C6 cells were inhibited, in a concentration-dependent fashion, and to a partial extent by WIN 55,212–2, a dual CB_1 and CB_2 receptor agonist. HIV-1 Tat-induced nitrite production was also partially inhibited by ACEA, a selective CB_1 agonist, but not by JWH-015, a selective CB_2 agonist. Furthermore, WIN 55,212–2-induced inhibition of both iNOS protein expression and nitrite production was reversed by SR141716A, a CB_1 receptor antagonist, whereas it resulted unaffected by the CB_2 receptor antagonist, SR1445528. These data indicate that the inhibition

of iNOS protein expression and nitrite production in C6 cells stimulated by HIV-1 Tat is selectively mediated by CB_1 receptor activation.

Our results support our previous suggestion (16) that the inhibition by cannabinoids of iNOS protein expression and nitrite in lipopolysaccharide-treated C6 cells is selectively mediated by CB_1 receptors (28). In the present study we also observed that HIV-1 Tat was toxic to C6 cells. Previously, Gavril *et al.* (49) showed that HIV-Tat protein was toxic to differentiated PC12 cells, a rat neuronal cell-line. Therefore, our results extend our knowledge of HIV-1 Tat cytotoxic effects also to a glial-derived cell-line. More importantly, we found that treatment of C6 cells with WIN 55,212–2 resulted in a potent protective action against cell death induced by HIV-1 Tat. This effect of WIN 55,212–2 appears to be selectively mediated by CB_1 receptors because it was attenuated by a CB_1, but not CB_2, receptor selective antagonist. We suggest that stimulation of CB_1 receptors leads to inhibition of HIV-1 Tat-induced cytotoxicity through the inhibition of the HIV-1 Tat-induced overproduction of NO, which has been found to have a toxic action on C6 cells (50, 51). Because, glial cell activation seems to play an important role in neurodegeneration (37–39), through the release of several cytotoxic mediators (40, 41), including NO (28), it can be hypothesized that cannabinoids, by inhibiting iNOS protein expression and NO overproduction, may protect neurons from HIV-1 Tat-induced damage. Such an effect, in the long term, may eventually result in a possible beneficial effect on AIDS dementia complex.

In this study we also present unprecedented evidence on the regulation of the endocannabinoid system by HIV-1 Tat. We found that HIV-1 Tat treatment of C6 cells down-regulates the CB_1, but not CB_2, receptor at the transcriptional level. This effect is probably related to HIV-1 Tat-induced overproduction of NO because the NO donor GSNO reduced CB_1 receptor expression in C6 cells. Because we have shown here that CB_1 receptor stimulation leads to an inhibition of HIV-1 Tat-induced NO release, it is possible that CB_1 receptor agonists also reduce HIV-1 Tat-induced down-regulation of CB_1 receptor expression. We could not test this hypothesis because long-term stimulation of CB_1 receptors is known to lead to their down-regulation and desensitization through several mechanisms (52, 53). Therefore, it would not have been easy to interpret measures of CB_1 receptor expression after 24 h co-incubation of C6 cells with HIV-1 Tat and WIN 55,212–2 (or ACEA).

In view of the multi-faceted neuroprotective effects exerted by the stimulation of CB_1 receptors (24), in many cell lines including C6 cells (54), we can speculate that part of HIV-1 Tat-induced neuronal damage is due to down-regulation of a neuroprotective endocannabinoid tone. However, the possible neurotoxic effect of HIV-1 Tat-induced down-regulation of CB_1 receptors might be minimized by HIV-1 Tat-induced inhibition of the cellular uptake and consequent degradation of the endocannabinoid anandamide by C6 cells, which was shown here for the first time. It is noteworthy that this effect of HIV-1 Tat, unlike the other effects described in this study, does not appear to be correlated to HIV-1 Tat-induced NO overproduction because NO has been found to slightly enhance, rather than inhibit, endocannabinoid cellular uptake in C6 cells (46, 52). Thus, it is conceivable that the increase of NO release by HIV-1 Tat prevented us from observing an even higher inhibitory action by HIV-1 Tat on anandamide reuptake. At any rate, even a small inhibitory effect of HIV-1 Tat on anandamide re-uptake by C6 cells is likely to result in a significant enhancement of the extracellular amounts of endocannabinoids available for CB_1 stimulation and, if occurring also in glial cells *in vivo*, might compensate in part for the down-regulation of CB_1 expression by HIV-1 Tat.

In conclusion, here we have presented for the first time data indicating that the endocannabinoid system protects C6 glioma cells from HIV-1 Tat-induced overproduction of NO and cell damage. Moreover, here we show for the first time that HIV-1 Tat inhibits CB_1 receptor expression and increases the amounts of extracellular endocannabinoids, thus regulating the levels of at least two components of the endocannabinoid system. Future *in vivo* studies should be addressed at establishing the relevance of these findings to the pathological effects of HIV-1 infection in the central nervous system, including AIDS dementia complex.

Acknowledgements—We thank Isabel Matias, Luciano De Petrocellis, and Maria Grazia Cascio for assistance.

REFERENCES

1. Jones, K. A. (1993) *Curr. Opin. Cell Biol.* **5,** 461–468
2. Chang, H. K., Gallo, R. C., and Ensoli, B. (1995) *J. Biomed. Sci.* **2,** 189–202
3. Watson, K., and Edwards, R. J. (1999) *Biochem. Pharmacol.* **58,** 1521–1528
4. Ensoli, B., Bonaguro, L., Barillari, G., Fiorelli, B., Gentleman, R., Morgan, R. A., Wingfield, P., and Gallo, R. C. (1993) *J. Virol.* **67,** 277–287
5. Tardieu, M., Hery, C., Peudenier, S., Boespflug, O., and Montagnier, L. (1992) *Ann. Neurol.* **32,** 11–17
6. Epstein, L. G., Gendelman, H. E., and Lipton, S. A. (1997) in *AIDS and the Nervous System* (Berger, J. R., and Levy, R. M., eds) 2nd, pp. 59–75, Lippincott-Raven Publishers, Philadelphia
7. Orsini, M. J., Debouck, C. M., Webb, C. L., and Lysko, P. G. (1996) *J. Neurosci.* **16,** 2546–2552
8. New, D. R., Maggirwar, S. B., Epstein, L. G., Dehwurst, S., and Gelbard, H. A. (1998) *J. Biol. Chem.* **273,** 17852–17858
9. Navia, B. A., Jordan, B. D., and Price, R. W. (1986) *Ann. Neurol.* **19,** 517–524
10. Price, R. W., Brew, B., Sidtis, J., Rosenblum, M., Scheck, A. C., and Cleary, P. (1988) *Science* **239,** 586–592
11. Mechoulam, R. (1986) in *Cannabinoids as Therapeutic Agents* (Mechoulam, R., ed) pp. 1–19, CRC Press, Boca Raton, FL
12. Pertwee, R. G. (1997) *Pharmacol. Ther.* **74,** 129–180
13. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., and Bonner, T. I. (1990) *Nature* **346,** 561–564
14. Munro, S., Thomas, K. L., and Abu-Shaar, M. (1993) *Nature* **365,** 61–65
15. Galiegue, S., Mary, S., Marchand, J., Dussosoy, D., Carriere, D., Carayon, P., Bouaboula, M., Shire, D., Le Fur, G., and Casellas, P. (1995) *Eur. J. Biochem.* **232,** 54–61
16. Galve-Ropher, I., Sanchez, C., Cortes, M. L., Gomez del Pulgar, T., Izquierdo, M., and Guzman, M. (2000) *Nat. Med.* **6,** 313–319
17. Devane, W. A., Hanus, L., Breuer, A., Pertewee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) *Science* **258,** 1946–1949
18. Mechoulam, R., Ben Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Shatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R., Pertewee, R. G., Griffin, G., Bayewitch, M., Barg, J., and Vogel, Z. (1995) *Biochem. Pharmacol.* **50,** 83–90
19. Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinode, A., Itoh, K., Yamashita, A., and Waku, K. (1995) *Biochem. Biophys. Res. Commun.* **215,** 89–97
20. Di Marzo, V., Melck, D., Bisogno, T., and De Petrocellis, V. (1998) *Trends Neurosci.* **21,** 521–528
21. Jin, K. L., Mao, X. O., Goldsmith, P. C., and Greenberg, D. A. (2000) *Ann. Neurol.* **48,** 257–261
22. Hansen, H. H., Schmid, P. C., Bittigau, P., Lastres-Becker, I., Berrendero, F., Manzanares, J., Ikonomidou, C., Schimid, H. H., Fernandez-Ruiz, J. J., and Hansen, H. S. (2001) *J. Neurochem.* **78,** 1415–1427
23. Panikshavili, D., Simenoidou, C., Ben Shabat, S., Hanus, L, Breuer, A., Mechoulam, R., and Shohami, E. (2001) *Nature* **413,** 527–531
24. Mechoulam, R., Spatz, M., and Shohami, E. (2002) *Sci. STKE* **129,** RE5
25. Coffey, R. G., Yamamoto, Y., Snella, E., and Pross, S. (1996) *Biochem. Pharmacol.* **52,** 743–751
26. Jeon, Y. J., Yang, K. H., Pulaski, J. T., and Kaminski, N. E. (1996) *Mol. Pharmacol.* **50,** 334–341
27. Waksman, J., Olson, J. M., Carlisle, J., and Cabral, G. A. (1999) *J. Pharmacol. Exp. Ther.* **288,** 1357–1366
28. Esposito, G., Izzo, A. A., Di Rosa, M., and Iuvone, T. (2001) *J. Neurochem.* **78,** 835–841
29. Knowles, R. G., Palacios, M., Palmer, R. M. J., and Moncada, S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5159–5162
30. Murphy, S., Minor, R. L., Jr., Welk, G., and Harrison, D. G. (1990) *J. Neurochem.* **55,** 349–351
31. Galea, E., Feinstein, D. L., and Reis, D. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10945–10949
32. Mollace, V., Colasanti, M., Rodino, P., Massoud, R., Lauro, G. M., and Nisticò, G. (1993) *Biochem. Biophys. Res. Commun.* **191,** 327–334
33. Garthwaite, J. (1991) *Trends Neurosci.* **14,** 60–67
34. Dawson, T. M., and Snyder, S. H. (1994) *J. Neurosci.* **14,** 5147–5159
35. Dawson, V. L., and Dawson, T. M. (1996) *Proc. Soc. Exp. Biol. Med.* **211,** 33–40
36. Bolanos, J. P., Almeida, A., Stewart, V., Peuchen, S., Land, J. M., Clark, J. B., and Heales, S. J. R. (1997) *J. Neurochem.* **68,** 2227–2240

37. Meda, L., Cassatela, M. A., Szendrei, G. I., Ottvos, L., Jr., Baron, P., Villalba, M., Ferrari, D., and Rossi, F. (1995) *Nature* **374,** 647–650
38. Chung, H., Brazil, M. I., Soe, T. T., and Maxfield, F. R. (1999) *J. Biol. Chem.* **274,** 32301–32308
39. Vila, M., Jackson-Lewis, V., Guegan, C., Wu, D. C., Teismann, P., Choi, D. K., Tieu, K., and Przedborski, S. (2001) *Curr. Opin. Neurol.* **14,** 483–489
40. Buttini, M., Appel, K., Sauter, A., Gebicke-Haerter, P. J., and Boddeke, H. W. (1996) *Neurosci.* **71,** 1–16
41. Rothwell, N. Toulmond, S., Allan, S., Stroemer, P., and Davies, C. (1999) in *Inflammatory Cells and Mediators in CNS Diseases* (Ruffolo, R. R., Feuersteiner, G. Z., Hunter, A. J., Poste, G., and Metcalf, B. W., eds) pp. 471–482, Harwood Academic Publisher, Canada
42. Ruvo, M., Scarallo, A., Vecchio, G., Palombo, G., and Fassina, G. (1996) in *Peptides* (Ramage, R., and Epton, R., eds) pp. 771–772, Mayflower Scientific, Ltd., Kingswinford West Midlands, UK
43. Polazzi, E., Levi, G., and Minghetti, L. (1999) *J. Neuropathol. Exp. Neurol.* **58,** 825–831
44. Di Rosa, M., Radomski, R. M., Carnuccio, R., and Moncada, S. (1990) *Biochem. Biophys. Res. Comm.* **172,** 1246–1252
45. Esposito, G., Aiello, A., Carbonelli, S., Menna, M. L., Fattorusso, E., and Iuvone, T. (2002) *Anticancer Res.* **22,** 2827–2832
46. Bisogno, T., Maccarrone, M., De Petrocellis, L., Jarrahian, A., Finazzi-Agro, A., Hillard, C., and Di Marzo, V. (2001) *Eur. J. Biochem.* **268,** 1982–1989
47. Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L., and Di Marzo, V. (1997) *J. Biol. Chem.* **272,** 3315–3323
48. Adamson, D. C., Wildemann, B., Sasaki, M., Glass, J. D., McArtur, J., Christov, V. I., Dawson, T. M., and Dawson, V. L. (1996) *Science* **274,** 1917–1921
49. Gavril, E. S., Cooney, R., and Weeks, B. S. (2000) *Biochem. Biophys. Res. Comm.* **267,** 252–256
50. Kim, H., Kim, Y. S., Kim, S. Y., and Suk, K. (2001) *Neurosci. Letts* **309,** 67–71
51. Suk, K., Kim, S. Y., and Kim, H. (2002) *J. Neurochem.* **80,** 230–238
52. Rinaldi-Carmona, M., Le Duigou, A., Oustric, D., Barth, F., Bouaboula, M., Carayon, P., Casellas, P., and Le Fur, G. (1998) *J. Pharmacol. Exp. Ther.* **287,** 1038–1047
53. Jin, W., Brown, S., Roche, J. P., Hsieh, C., Celver, J. P., Kovoor, A., Chavkin, C., and Mackie, K. (1999) *J. Neurosci.* **19,** 3773–3780
54. Maccarrone, M., Bari, M., Lorenzon, T., Bisogno, T., Di Marzo, V., and Finazzi-Agro, A. (2000) *J. Biol. Chem.* **275,** 13484–13492